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Occurrence and Evolutionary History of Two *Cynops pyrrhogaster* Lineages on the Izu Peninsula

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Abstract: We investigated phylogenetic positions of *Cynops pyrrhogaster* from nine localities in the central and southern parts of the Izu Peninsula using the mitochondrial cyt b gene. We revealed that the central and the southern populations are phylogenetically remote. The central Izu lineage belongs to the CENTRAL clade occurring from Chubu through Kinki to Chugoku districts, whereas the southern Izu populations form a lineage sister to the NORTHERN clade, which is distributed in Tohoku and Kanto districts. Genetic differentiation between the southern Izu lineage and the NORTHERN clade is relatively large with the uncorrected p-distance of 3.4%, which suggests their divergence at 3.31 MYA. This estimation indicates their genetic differentiation prior to 1.0 MYA, when the Izu Peninsula was formed through collision of a paleo-oceanic island with Honshu. These results indicate that the ancestor of the southern Izu lineage diverged from the NORTHERN clade somewhere in northern Honshu and then invaded the Izu Peninsula newly formed by collision and settled there. The central Izu lineage thereafter also invaded the peninsula, confining the range of the preceding southern Izu lineage to its current range.

Key words: *Cynops*; Peripheral population; Izu Peninsula; Mitochondrial DNA; Phylogeography

Introduction

Many studies using molecular techniques, which have experienced great progress recently, have revealed the presence of cryptic genetic divergence among populations of various animals that are morphologically weakly divergent. These studies have found that many isolated populations and those from peripheral areas of the range show extensive genetic differentiation from the other populations (e.g., Yoshikawa et al., 2008; Tominaga et al., 2014).

The Japanese fire-bellied newt, *Cynops pyrrhogaster*, occurs all over Japan except for Hokkaido and Okinawa Prefectures. This species has been known to show extensive morphological, behavioral, and genetic varia-

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tion (Sawada, 1963a, b; Hayashi and Matsui, 1988, 1990; Tominaga et al., 2013). Tominaga et al. (2013) revealed that this species includes four major genetic clades, i.e., the NORTHERN, CENTRAL, WESTERN, and SOUTHERN clades, which show parapatric distributions. Unfortunately, sample sizes from some localities in their study were small and additional intensive sampling was awaited. Among them, the sample from the Izu Peninsula was represented by only one specimen from the central part of the peninsula. In Tominaga et al. (2013), this specimen belonged to the CENTRAL clade, which ranges from Chubu to eastern Chugoku district, but because of this limited sample size, fine-scale genetic structure on this peninsula remained unclarified. Subsequently, we collected additional specimens from the peninsula and found an interesting distributional pattern of haplotypes, suggesting diverse origins.

Materials and Methods

Sampling and data used in this study

We collected 57 new specimens from nine localities on the Izu Peninsula (Fig. 1; Table 1). Total DNA was extracted from ethanol-preserved tissues using standard phenol-chloroform extraction procedures (Hillis et al., 1996). The fragments containing NADH6, $tRNA^{glu}$, and cytochrome b (cyt b) genes (total 1393 bp) were amplified by PCR for six representing specimens from three localities. We additionally sequenced partial cyt b gene (748 bp) for the remaining 51 specimens to

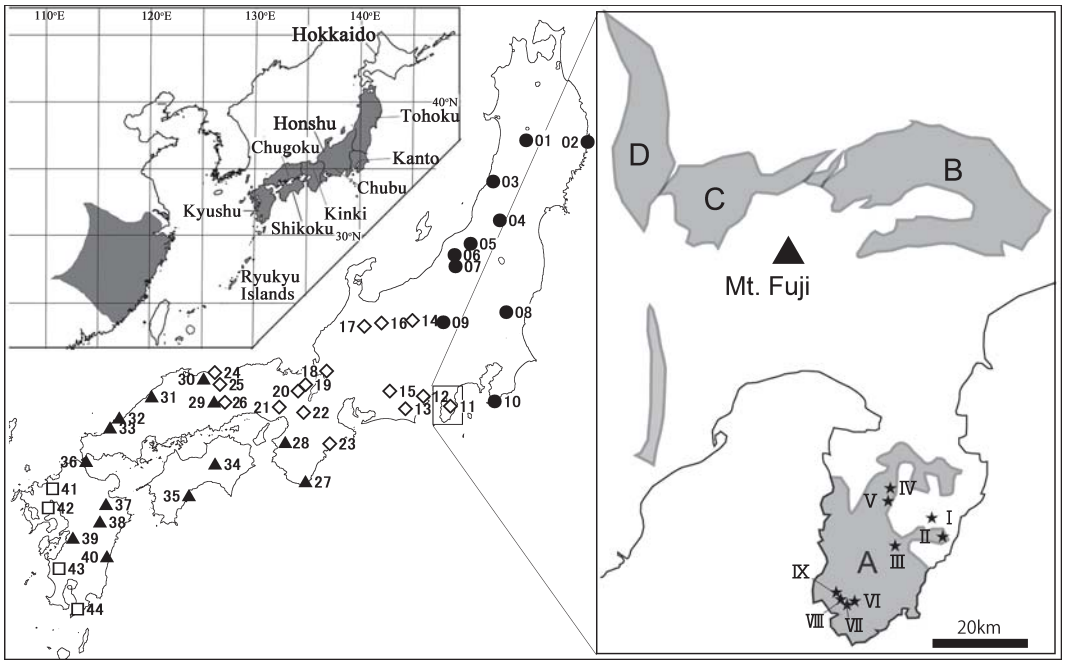

Fig. 1. Map of Japan showing sampling localities and distribution ranges of *Cynops pyrrhogaster* and other *Cynops* species (Gray zones in the East Asian Map). Closed circle: NORTHERN clade; Open diamond: CENTRAL clade; Closed triangle: WESTERN clade; Open square: SOUTHERN clade; Closed star in the map of the Izu Peninsula: localities for specimens newly obtained in this study. Arabic numbers adjacent to the locality marks indicate the locality numbers for specimens analyzed by Tominaga et al. (2013). Roman numbers adjacent to closed stars refer to the locality numbers for specimens obtained in this study. These locality numbers correspond to those in Figs. 2 and 3 and Table 1. Gray zones in the map of the Izu Peninsula indicate paleo-oceanic land-brock distributions. A: Izu land-block; B: Tanzawa land-block; C: Misaka land-block; D: Kushigatayama land-block. Data taken from Amano et al. (2007).

TABLE 1. Locality numbers, locality name, number of specimens, and haplotype compositions in each locality for newly obtained specimens in this study.

Locality number	Locality name	N of specimens	Haplotype based on partial cyt b
I	Higashiizu 1	5	e (2), f (3)
II	Higashiizu 2	6	e (3), f (3)
III	Kawazu	6	e (5), f (1)
IV	Izu 1	3	e (3)
V	Izu 2	6	e (6)
VI	Minamiizu 1	9	a (2), b (5), d (2)
VII	Minamiizu 2	8	a (3), b (1), d (4)
VIII	Minamiizu 3	5	a (2), d (3)
IX	Minamiizu 4	9	a (3), b (3), c (3)

decide their genetic lineages. The sequencing was performed on ABI 3100 or ABI 3130 automatic sequencers. The detailed experimental procedure and primers used in this study are the same as those reported in Tominaga et al. (2013). The obtained sequences were deposited in the DDBJ under accession numbers LC016764–LC016772.

For phylogenetic analysis, we added total 65 sequences from DDBJ, which represent part of the dataset in Tominaga et al. (2013). Alignment of data from all sequences was performed using the Clustal option in the BioEdit software (Hall, 1999).

Phylogenetic analyses

We constructed phylogenetic trees by maximum likelihood (ML), Bayesian (BI), and maximum parsimony (MP) methods. Prior to phylogenetic analyses, the dataset of mtDNA was divided into seven partitions: each of the three codon positions (1st, 2nd, and 3rd positions) of NADH 6 and cyt b genes, and $tRNA^{glu}$ gene. The optimum substitution models for each partition were selected by Kakusan4 (Tanabe, 2011), based on the Akaike information criterion. The ML tree was searched using TREEFINDER ver. Oct. 2008 (Jobb et al., 2004; Jobb, 2008) and Phylogears2 (Tanabe, 2008) through 100 trials of likelihood ratchet method (Vos, 2003). The Bayesian analysis was conducted using MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). Two independent runs of four Markov chains were conducted for three million generations in the Bayesian analyses. We sampled one tree every 100 generations and calculated a consensus topology for 27,000 trees after discarding the first 3,001 trees (burn-in=300,001). Parameter estimates and convergence were checked using Tracer version 1.4 (Rambaut and Drummond, 2007). The MP tree was constructed using PAUP* 4.0b10 (Swofford, 2002). MP phylogenies were estimated using the heuristic search algorithm for each tree-building methodology. We used 100 random-taxon-addition replicates for all analyses to minimize the effect of entry sequence on the topology of the resulting cladogram. We conducted the analyses with accelerated character transformation (ACCTRAN) optimization and tree bisection-reconnection (TBR) branch swapping, with characters unordered and equally weighted. For the ML and MP analyses, non-parametric bootstrap (bs) analysis (Felsenstein, 1985) with 1,000 replicates was used. Branches with bootstrap values 70% or greater were regarded as sufficiently resolved (Huelsenbeck and Hillis, 1993). For the Bayesian analysis, posterior probabilities (bpp) were used as an indicator of node credibility, and those 95% or greater were considered significant (Leaché and Reeder, 2002).

To identify the genetic lineages for the remaining 51 specimens, we added their partial cyt b sequences to the dataset as above and conducted neighbor joining analysis based on Kimura's two parameter distance (Kimura, 1980) with 1,000 bootstrap (bs) analysis using MEGA, version 4 (Tamura et al., 2007).

Calculation of genetic distance and estimation of divergence time

We calculated uncorrected p-distances based on the cyt b gene for pairwise combinations of haplotypes using MEGA, version 4. A time-calibrated phylogeny was estimated based on mtDNA data using BEAST (Drummond and Rambaut, 2007), which permits simultaneous

Bayesian estimation of phylogeny and divergence times under a non-autocorrelated relaxed clock model. The BEAST analysis was run for 10 million generations under a HKY85+G, TN93+G, and HKY85+G substitution model for NADH6, $tRNA^{glu}$, and cyt b genes, respectively, and an uncorrelated log-normal "relaxed" clock rate model (Drummond et al., 2006). The MCMC chain was sampled every 1,000 generations, for a total of 10,001 samples, and convergence with the stationary distribution was assessed through inspection of the likelihood and parameter sample plots in Tracer; a burn-in of 1,000 samples was adopted. Large effective sample sizes (ESS) of all parameters across the post burn-in chain samples confirmed that the MCMC chain was mixing well. We used two independent calibration points: A *Triturus* fossil dated at 24 MYA was interpreted as approximating the crown of the genus *Triturus*, following Wielstra et al. (2010) and Steinfartz et al. (2007). The divergence time of three Asian genera (*Paramesotriton*, *Pachytriton*, and *Cynops*) was set at 16MYA, based on the dating of Larson et al. (2003).

Results

Phylogenetic relationships among specimens

For the ML analysis, HKY85 (Hasegawa et al., 1985) model, HKY85+G, J2 (Jobb, 2008)+I+G, TVM (Posada, 2003)+I, HKY85+G, HKY85+G, and J2+I+G were selected as the optimal models for 1st, 2nd, and 3rd codon positions of ND6, $tRNA^{glu}$, 1st, 2nd, and 3rd codon positions of cyt b genes, respectively. For the Bayesian analyses, F81 (Felsenstein, 1981), HKY85+G, HKY85+I+G, GTR (Tavaré, 1986)+I, HKY85+G, HKY85+G, and GTR+I+G were selected as the best substitution model for 1st, 2nd, and 3rd codon positions of ND6, $tRNA^{glu}$, 1st, 2nd, and 3rd codon positions of cyt b genes, respectively.

In the phylogenetic analyses based on the full sequence data set, we detected three new haplotypes identified (Fig. 2). Of these, two were detected from specimens from the southern part of the peninsula, and the remaining one was from central part of the peninsula. Phylogenetic analyses confirmed that *C. pyrrhogaster* includes four previously recognized clades (the NORTHERN, CENTRAL, WESTERN, and SOUTHERN clades; Tominaga et al., 2013). The phylogenetic pattern among the four clades was also same as that determined by Tominaga et al. (2013), although monophyly of the CENTRAL, WESTERN, and SOUTHERN clades was supported only by MP inference (45/0.91/75 in ML bs/bpp/MP bs).

A haplotype detected from the central part of the Izu Peninsula formed a lineage (hereafter referred to as the central Izu lineage) with the single specimen from the Izu Peninsula used by Tominaga et al. (2013) and was nested in the CENTRAL clade. On the other hand, two haplotypes from southern part of the peninsula were closer to the NORTHERN clade than to the CENTRAL clade, and formed a lineage (hereafter referred to as the southern Izu lineage) sister to the NORTHERN clade. A monophyletic relationship between the southern Izu lineage and the NORTHERN clade was strongly supported (99/1.00/100).

Of the 51 additional specimens, all of the 28 specimens from the southern Izu Peninsula were identified as the southern Izu lineage, and four haplotypes (haplotypes a, b, c, and d) were detected within this lineage (Fig. 3; Table 1). On the other hand, all of the 23 specimens from the central Izu Peninsula were identified as the central Izu lineage, and two haplotypes (haplotypes e and f) were observed in the lineage (Fig. 3; Table 1).

Genetic distance and estimated divergence times

The genetic distances among and within clades and lineages are shown in Table 2. The uncorrected p-distance between the southern Izu lineage and the NORTHERN clade is 3.4±0.8%. Divergence-time estimation revealed overlap of 95% HPD on estimates of divergence times in major clades of Japanese newts

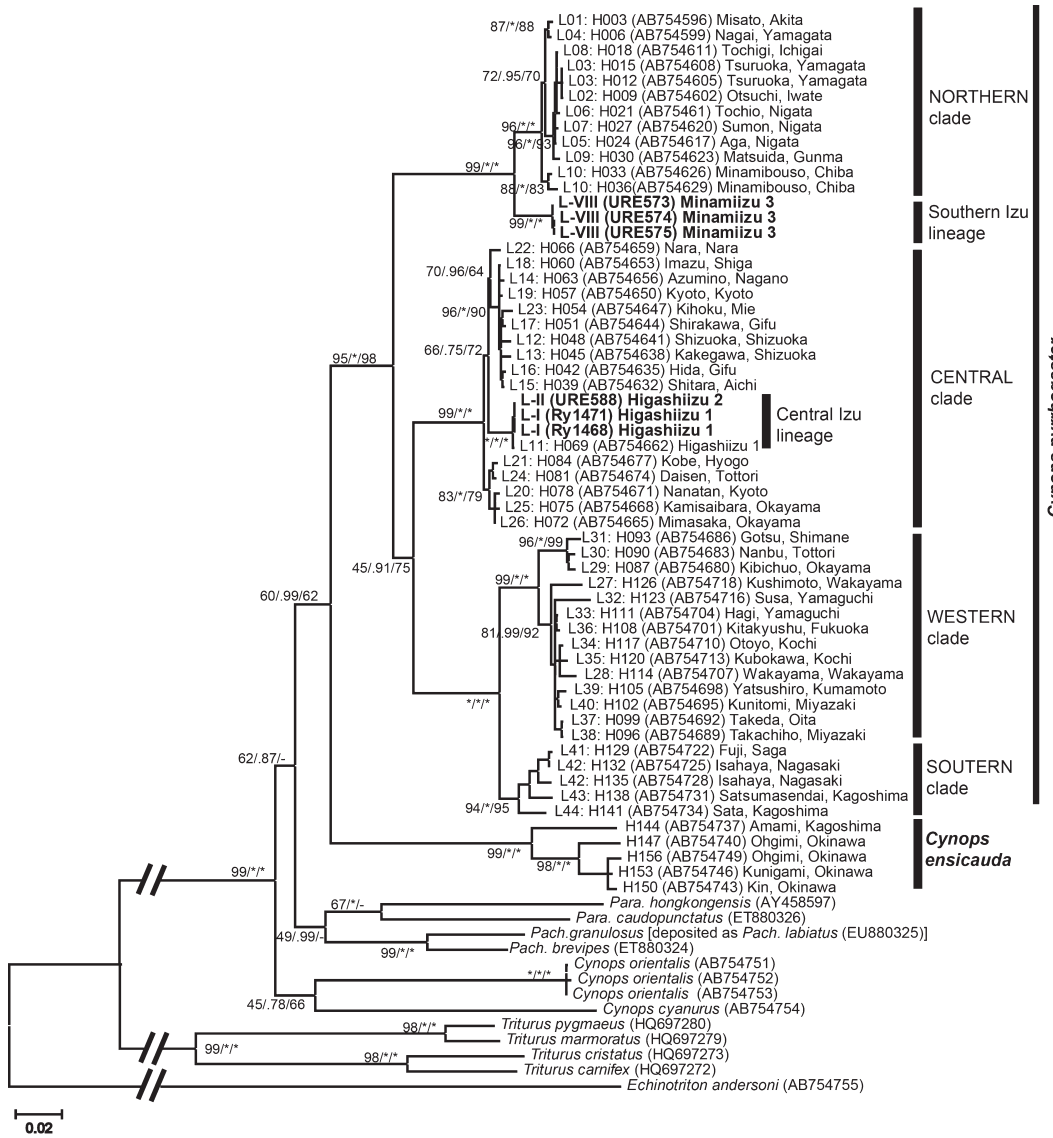

FIG. 2. Maximum likelihood phylogram of 1393 bp of mitochondrial genes for samples of *Cynops pyrrhogaster* and its related species. Numbers preceded by "L" indicate locality number and those preceded by "H" are haplotype number designated by Tominaga et al. (2013). Numbers in parentheses indicate DDBJ accession numbers. Nodal numbers represent ML bootstrap supports/Bayesian posterior probability/MP bootstrap supports. Asterisks indicate 100% bootstrap support values or 1.00 Bayesian posterior probabilities.

(Table 2). Most divergence times among and within clades or lineages were nearly same with those estimated by Tominaga et al. (2013). The times of divergence between the NORTHERN clade and the southerrn Izu lineage was 3.31 (95% HPD 1.83–5.14) MYA.

DISCUSSION

Phylogenetic positions of specimens from the Izu Peninsula

Tominaga et al. (2013) reported that *C. pyrrhogaster* is composed of four major clades (the NORTHERN, CENTRAL, WESTERN,

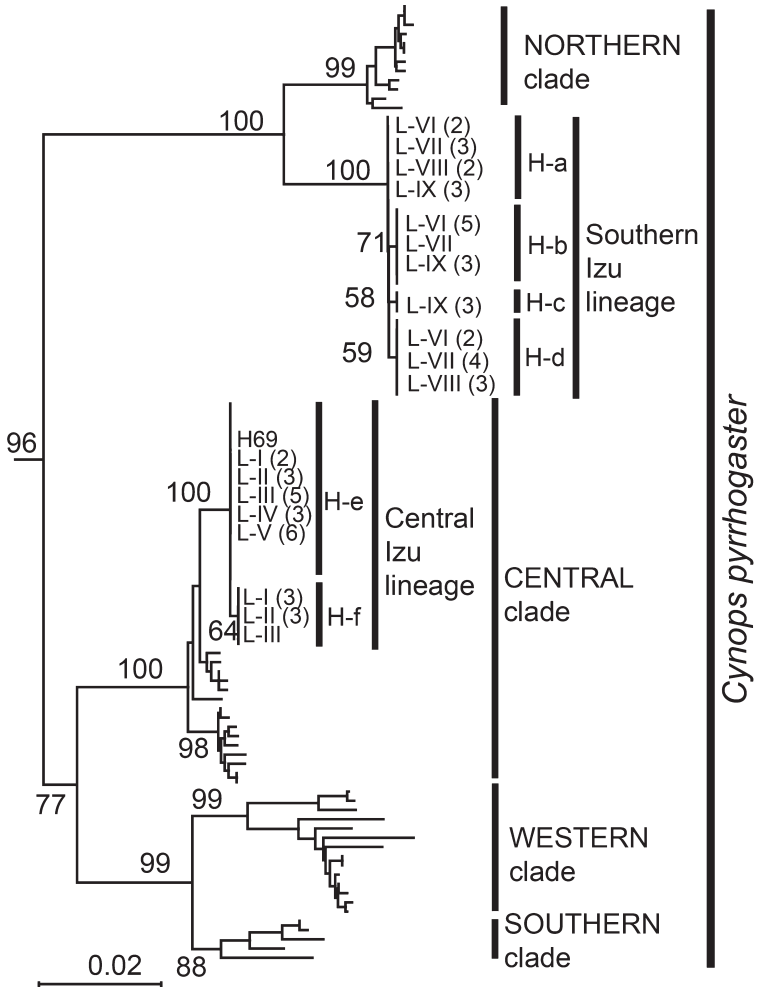

FIG. 3. Neighbor joining tree based on the partial cyt b sequences. Roman numbers preceded by "L" indicate locality number and alphabets preceded by "H" are haplotypes designated in this study. Numbers in parentheses indicate number of specimens. Nodal numbers represent NJ bootstrap supports.

and SOUTHERN clades). The specimen from the central Izu Peninsula used by Tominaga et al. (2013) was nested in the CENTRAL clade. Together with that specimen, all 26 newly obtained specimens from central part of the peninsula were included in the CENTRAL clade. On the other hand, all the remaining 31 specimens from southern part of the peninsula formed a sister lineage (southern Izu lineage) to the NORTHERN clade, but the genetic distance between them was quite large (3.4±0.8% in cyt b). This result indicates that the southern Izu lineage diverged from the NORTHERN clade, but is currently geographically isolated from the latter clade and confined to the southern part of the peninsula, with the intervening northern to central areas of the peninsula occupied by the central Izu lineage of the CENTRAL clade. Our intensive field surveys revealed that these two lineages are parapatrically distributed without co-occurrence on the peninsula. To further clarify the genetic and taxonomic relationships among them, of which morphological features are different between the lineages (Tominaga et al. unpulished data), investiga-

TABLE 2. Genetic distance and estimated divergence times (MYA) of main divergences among and within clades and lineages based on mitochondrial DNA. Asterisks indicate calibration points.

Combination	K2P distance (±SE) in cyt b	Uncorrected p-distance (±SE) in cyt b	Mean of divergence time and 95% HPD in parentheses
Triturus vs. three Asian genera	26.5±1.3	21.7±0.9	36.24 (28.46–44.72)
Triturus (*T. carnifex*+*T. cristatus* vs. *T. marmoratus*+*T. pygmaeus*)	16.9±1.1	14.7±0.9	23.26 (21.31–25.20)*
Three Asian genera	14.1±0.8	12.5±0.6	16.56 (14.69–18.38)*
C. pyrrhogaster vs. *C. ensicauda*	14.4±0.9	12.7±0.7	13.68 (11.14–16.17)
C. pyrrhogaster (NORTHERN+Southern Izu lineage vs. other three clades)	10.2±0.8	9.3±0.7	9.93 (7.47–12.39)
C. ensicauda (Amami vs. Okinawa)	7.0±0.8	6.6±0.7	5.87 (3.71–8.14)
CENTRAL vs. WESTERN+SOUTHERN	8.0±0.7	7.5±0.7	8.55 (6.29–11.05)
WESTERN vs. SOUTHERN	4.6±0.5	4.4±0.5	4.63 (3.11–6.20)
NORTHERN clade vs. Southern Izu linegae	3.5±0.5	3.4±0.8	3.31 (1.83–5.14)
Central Izu lineage vs. the closest linegae within CENTRAL clade	1.7±0.3	1.6±0.3	1.63 (0.91–2.50)
Within NORTHERN Clade	0.8±0.2	0.8±0.2	1.44 (0.78–2.25)
Within CENTRAL Clade	1.2±0.2	1.2±0.2	2.01 (1.10–3.07)
Within WESTERN Clade	1.8±0.2	1.8±0.2	2.72 (1.73–3.90)
Within SOUTHERN Calde	1.6±0.2	1.5±0.2	2.61 (1.53–3.94)

tions using other molecular markers, especially nuclear genes, are needed.

Divergence time and hypothesized dispersal history

The Izu Peninsula is located at the northern tip of the Philippine Sea plate (Sugimura, 1972). This plate had intermittently transferred four paleo-oceanic islands (a part of the Izu-Bonin Arc) northwards, and these islands collided with and accreted to Honshu (Amano, 1991). These paleo-oceanic islands correspond to the current Kushigatayama land-block, the Misaka land-block, the Tanzawa land-block, and the Izu land-block, which are distributed around this region (Fig. 1). The current Izu Peninsula is derived from a paleo-oceanic island that had collided with Honshu about 1 MYA (Amano et al., 2007).

The divergence time estimated between the NORTHERN clade and the southern Izu lineage was 3.31 (95% HPD 1.83–5.14) MYA, which is much older than the age when the Izu Peninsula collided with Honshu. This estimation suggests that the ancestor of the southern Izu lineage diverged from the NORTHERN clade somewhere in northern Honshu and thereafter invaded the peninsula, which was newly formed by collision after 1 MYA. The divergence time between them (3.31 [1.83–5.14] MYA) roughly coincides with the age of the collision of the third paleo-oceanic island, which corresponds to the current Tanzawa land-block (5 MYA). Because the available geo-historical information is limited for this era, we cannot specify the event that caused the differentiation between the NORTHERN clade and the southern Izu lineage, but the collision of the Tanzawa land-block might have affected their divergence.

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